EXHIBIT 2

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Exhibit 2

Toward a code for the interactions of zinc fingers with DNA: Selection of randomized fingers displayed on phage

(recognition code/DNA–protein interaction/protein design)

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ABSTRACT We have used two selection techniques to study sequence-specific DNA recognition by the zinc finger, a small, modular DNA-binding minidomain. We have chosen zinc fingers because they bind as independent modules and so can be linked together in a peptide designed to bind a predetermined DNA site. In this paper, we describe how a library of zinc fingers displayed on the surface of bacteriophage enables selection of fingers capable of binding to given DNA triplets. The amino acid sequences of selected fingers which bind the same triplet are compared to examine how sequence-specific DNA recognition occurs. Our results can be rationalized in terms of coded interactions between zinc fingers and DNA, involving base contacts from a few α-helical positions. In the paper following this one, we describe a complementary technique which confirms the identity of amino acids capable of DNA sequence discrimination from these positions.

The manner in which DNA-binding protein domains are able to discriminate between different DNA sequences is an important question in understanding crucial processes such as the control of gene expression in differentiation and development. The zinc finger motif has been studied extensively, with a view to providing some insight into this problem, owing to its remarkable prevalence in the eukaryotic genome and its important role in proteins which control gene expression in *Drosophila* (e.g., ref. 1), mice (2), and humans (3).

Most sequence-specific DNA-binding proteins bind to the DNA double helix by inserting an α-helix into the major groove (4–6). Sequence specificity results from the geometrical and chemical complementarity between the amino acid side chains of the α-helix and the accessible groups exposed on the edges of base pairs. In addition to this direct reading of the DNA sequence, interactions with the DNA backbone stabilize the complex and are sensitive to the conformation of the nucleic acid, which in turn depends on the base sequence (7). *A priori*, a simple set of rules might suffice to explain the specific association of protein and DNA in all complexes, based on the possibility that certain amino acid side chains have preferences for particular base pairs. However, crystal structures of protein–DNA complexes have shown that proteins can be idiosyncratic in their mode of DNA recognition because they use alternative geometries to present their sensory α-helices to DNA, allowing a variety of different base contacts to be made by a single amino acid and vice versa (8). Nevertheless, for a family of transcription factors which use a "probe helix" for binding to the major groove of DNA, it would seem possible to deduce some general principles (9).

We believe the zinc finger of the class found in transcription factor TFIIIA to be a good candidate for deriving a set of specificity rules, owing to its great simplicity of structure and interaction with DNA. The zinc finger is an independently folding domain which uses a zinc ion to stabilize the packing of an antiparallel β-sheet against an α-helix (10–12). The crystal structures of zinc finger–DNA complexes show a semiconserved pattern of interactions in which three amino acids from the α-helix contact three adjacent bases (a triplet) in DNA (13–15). Thus the mode of DNA recognition is principally a one-to-one interaction between amino acids and bases. Because zinc fingers function as independent modules (10, 16), fingers with different triplet specificities are combined to give specific recognition of longer DNA sequences. Protein engineering experiments have shown that it is possible to alter rationally the DNA-binding characteristics of individual zinc fingers when one or more of the α-helical positions are varied in a number of proteins (17–19). Because a large collection of these mutants is accumulating, it has already been possible to propose some rules relating amino acids on the α-helix to corresponding bases in the bound DNA sequence (20). However, in this approach the altered positions on the α-helix are prejudged, making it possible to overlook the role of positions which are not currently considered important; further, owing to the importance of context, concomitant alterations are sometimes required to affect specificity (20), so that a significant correlation between an amino acid and base may be misconstrued.

An alternative to the rational but biased design of proteins with new specificities is the isolation of desirable mutants from a large pool. A powerful method of selecting such proteins is the cloning of peptides (21), or protein domains (22, 23), as fusions to the minor coat protein (pIII) of bacteriophage fd, which leads to their expression on the tip of the capsid. Phage displaying the peptides of interest can then be affinity purified and amplified for use in further rounds of selection and for DNA sequencing of the cloned gene. We have applied this technology to the study of zinc finger–DNA interactions, after demonstrating that functional zinc finger proteins can be displayed on the surface of fd phage, and that the engineered phage can be captured on a solid support coated with specific DNA. A phage display library has been created comprising variants of the middle finger from the DNA-binding domain of Zif268 (a mouse transcription factor containing three zinc fingers) (2). DNA of fixed sequence is used to purify phage from this library over several rounds of selection, returning a number of different but related zinc fingers which bind the given DNA. By comparing similarities in the amino acid sequences of functionally equivalent fingers, we deduce the likely mode of interaction of these fingers with DNA. Remarkably, it would appear that many base contacts can occur from three primary positions on the α-helix of the zinc finger, correlating with the implications of the crystal structure of Zif268 bound to DNA (13). The ability to select or design zinc fingers with desired specificity means that in the near future, DNA-binding proteins containing zinc fingers will be made to measure.

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MATERIALS AND METHODS

Construction and Cloning of Genes. The gene for the first three fingers (residues 3–101) of transcription factor TFIIIA was amplified by PCR from the cDNA clone of TFIIIA, using forward and backward primers which contained restriction sites for *Not* I and *Sfi* I, respectively. The gene for the Zif268 fingers (residues 333–420) was assembled from eight overlapping synthetic oligonucleotides, giving *Sfi* I and *Not* I overhangs. The genes for fingers of the phage library were synthesized from four oligonucleotides by directional end-to-end ligation using three short complementary linkers and amplified by PCR from the single strand by using forward and backward primers which contained sites for *Not* I and *Sfi* I, respectively. Backward PCR primers in addition introduced Met-Ala-Glu as the first three amino acids of the zinc finger peptides, and these were followed by the residues of the wild-type or library fingers as discussed in the text. Cloning overhangs were produced by digestion with *Sfi* I and *Not* I where necessary. Fragments were ligated to 1 μg of similarly prepared Fd-Tet-SN vector. This is a derivative of fd-tet-DOG1 (24) in which a section of the *pelB* leader and a restriction site for the enzyme *Sfi* I (underlined) have been added by site-directed mutagenesis using the oligonucleotide 5′-CTCCTGCAGTTGGACCTGTGCC ATGGCCGGC-TGGGCCGCATAGAATGGAACAACTAAAGG-3′, which anneals in the region of the polylinker (L. Jespers, personal communication). Electrocompetent *Escherichia coli* DH5α cells were transformed with recombinant vector in 200-ng aliquots, grown for 1 hr in 2× TY medium (1.6% tryptone/1% yeast extract/0.5% NaCl) with 1% glucose, and plated on TYE medium (1.5% agar/1% Bacto-Tryptone/0.5% yeast extract/0.8% NaCl) containing tetracycline (15 μg/ml) and 1% glucose.

Phage Selection. Colonies were transferred from plates to 200 ml of 2×TY/Zn/Tet (2×TY containing 50 μM zinc acetate and 15 μg of tetracycline per ml) and grown overnight. Phage were purified from the culture supernatant by two rounds of precipitation using 0.2 volume of 20% polyethylene glycol/2.5 M NaCl/50 μM zinc acetate and resuspended in zinc-finger phage buffer (20 mM Hepes, pH 7.5/50 mM NaCl/1 mM $MgCl_2$/50 μM zinc acetate). Streptavidin-coated paramagnetic beads (Dynal, Oslo) were washed in zinc-finger phage buffer and blocked for 1 hr at room temperature with the same buffer made 6% (wt/vol) in fat-free dried milk (Marvel). Selection of phage was over three rounds: in the first round, beads (1 mg) were saturated with biotinylated oligonucleotide (≈80 nM) and then washed prior to phage binding, but in the second and third rounds 1.7 nM oligonucleotide and 5 μg of poly(dG-dC) (Sigma) were added to the beads with the phage. Binding reaction mixtures (1.5 ml) contained zinc-finger phage buffer with 2% (wt/vol) fat-free dried milk, 1% (vol/vol) Tween 20 and, typically, 5×10^{11} phage and were incubated for 1 hr at 15°C. Beads were washed 15 times with 1 ml of the same buffer. Phage were eluted by shaking in 0.1 M triethylamine for 5 min and neutralized with an equal volume of 1 M Tris (pH 7.4). Logarithmic-phase *E. coli* TG1 cells in 2×TY were infected with eluted phage for 30 min at 37°C and plated as described above. Phage yields were titred by plating serial dilutions of the infected bacteria.

DNA Sequence Analysis of Selected Phage. Single colonies of transformants obtained after three rounds of selection were grown overnight in 2×TY/Zn/Tet. Small aliquots of the cultures were stored in 15% (vol/vol) glycerol at −20°C, to be used as an archive. Single-stranded DNA was prepared from phage in the culture supernatant and sequenced with Sequenase 2.0 (United States Biochemical).

RESULTS AND DISCUSSION

Phage Display of Three-Finger DNA-Binding Domains from TFIIIA or Zif268. Prior to the construction of a phage display library, we demonstrated that peptides containing three functional zinc fingers could be displayed on the surface of viable fd phage when cloned in the vector Fd-Tet-SN. In preliminary experiments, we cloned as fusions to pIII first the three N-terminal fingers from TFIIIA (25) and second the three fingers from Zif268 (2), for both of which the DNA binding sites are known. Peptide fused to the minor coat protein was detected in Western blots with an anti-pIII antibody (26). Approximately 10–20% of total pIII in phage preparations was present as fusion protein.

Phage displaying either set of fingers were capable of binding to specific oligodeoxynucleotides, indicating that zinc fingers were expressed and correctly folded in both instances. Paramagnetic beads coated with specific oligonucleotide were used as a medium on which to capture DNA-binding phage (Fig. 1 *a* and *c*), and were consistently able to return between 100- and 500-fold more such phage, compared with free beads or beads coated with nonspecific DNA. Alternatively, when phage displaying the three fingers of Zif268 were diluted $1:1.7 \times 10^3$ with Fd-Tet-SN phage not bearing zinc fingers, and the mixture was incubated with beads coated with Zif268 operator DNA, one in three of the total phage eluted and transfected into *E. coli* were shown by colony hybridization to carry the Zif268 gene, indicating an enrichment factor of >500 for the zinc finger phage. Hence it is clear that zinc fingers displayed on fd phage are capable of preferential binding to DNA sequences with which they can form specific complexes, making possible the enrichment of wanted phage by factors of up to 500 in a single affinity-purification step. Therefore, over multiple rounds of selection and amplification, very rare clones capable of sequence-specific DNA binding can be selected from a large library.

A Phage Display Library of Zinc Fingers from Zif268. We have made a phage display library of the three fingers of Zif268 in which selected residues in the middle finger are randomized (Fig. 1*b*), and we have isolated phage bearing zinc fingers with desired specificity by using a modified Zif268 operator sequence (28) in which the middle DNA triplet is altered to the sequence of interest (Fig. 1*c*). To be able to study both the primary and secondary putative base recognition positions which are suggested by database analysis (29), we have designed the library of the middle finger so that positions −1 to +8 [relative to the first residue in the α-helix (position +1)], but excluding the conserved Leu and His, can be any amino acid except Phe, Tyr, Trp, and Cys, which occur rarely at those positions (30). In addition, we have allowed position +9 [which might make an inter-finger contact with Ser at position −2 (13)] to be either Arg or Lys, the two most frequently occurring residues at that position.

The logic of this protocol, based upon the Zif268 crystal structure (13), is that the randomized finger is directed to the central triplet, since the overall register of protein–DNA contacts is fixed by its two neighbors. This enables us to examine which amino acids in the randomized finger are the most important in forming specific complexes with DNA of known sequence. Since comprehensive variations are programmed in all the putative contact positions of the α-helix, we can conduct an objective study of the importance of each position in DNA binding (29).

The size of the phage display library required—if we assume full degeneracy of the eight variable positions—is $(16^7 \times 2^1 =) 5.4 \times 10^8$, but because of practical limitations in the efficiency of transformation with Fd-Tet-SN, we have been able to clone only 2.6×10^6 of these. The library we use is therefore some 200 times smaller than the theoretical size necessary to cover all the possible variations of the α-helix.

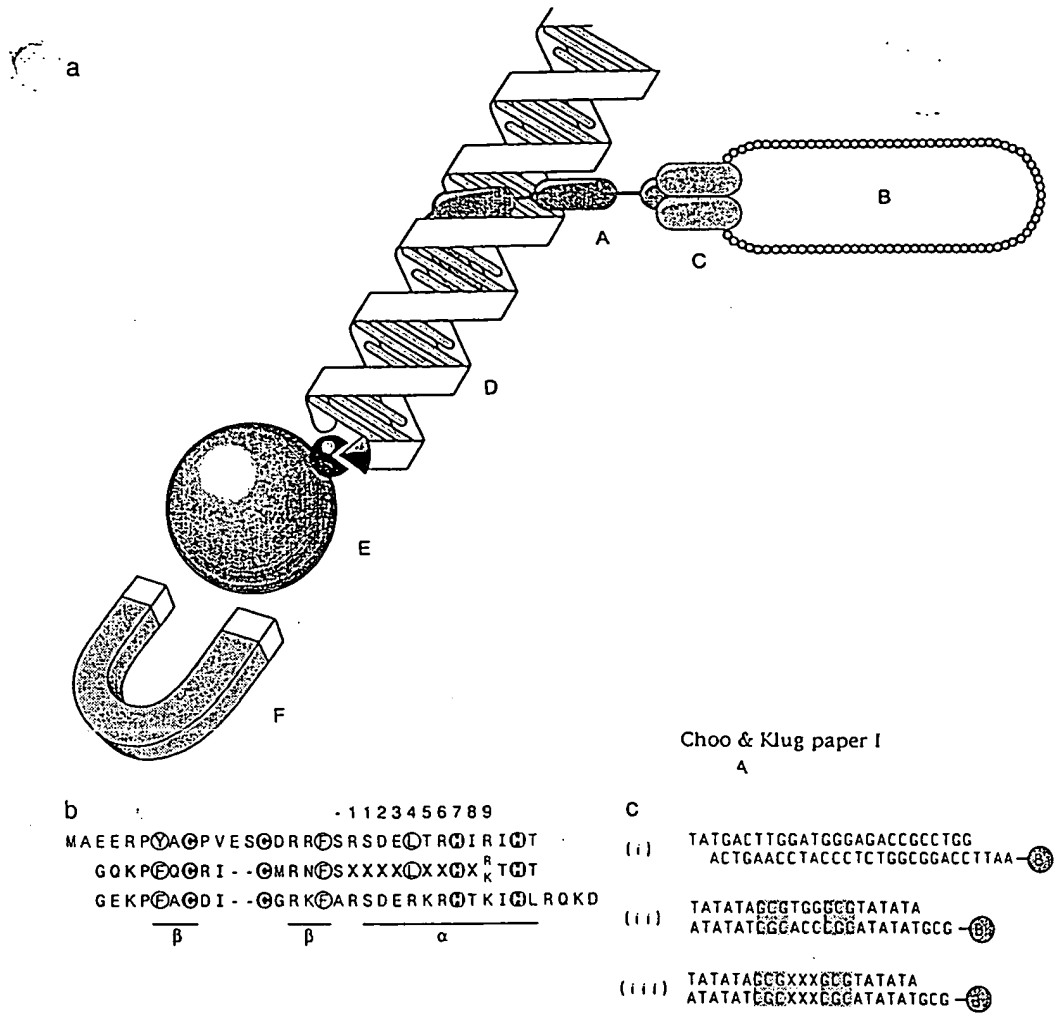

FIG. 1. Affinity purification of zinc finger phage. (*a*) Zinc fingers (A) are expressed on the surface of fd phage (B) as fusions to the minor coat protein pIII (C). Zinc finger phage are bound to 5'-biotinylated DNA oligonucleotide (D) attached to streptavidin-coated paramagnetic beads (E) and are captured with a magnet (F). [Figure adapted from the Dynal (Oslo) catalog and also Marks *et al.* (27).] (*b*) Amino acid sequence of the three zinc fingers from Zif268 used in the phage display library. The randomized positions in the α-helix of the second finger have residues marked X. The amino acid positions are numbered relative to the first helical residue (position +1). For amino acids at positions −1 to +8, excluding the conserved Leu and His, codons are equal mixtures of (G/A/C)NN; T in the first base position is omitted in order to avoid stop codons, but this has the effect that the codons for Trp, Phe, Tyr, and Cys are not represented. Position +9 is specified by the codon A(G/A)G, allowing either Arg or Lys. Residues of the hydrophobic core are circled, whereas the zinc ligands are written as white letters on black circles. The positions forming the β-sheets and the α-helix of a zinc finger are marked below the sequence. (*c*) Sequences of oligodeoxynucleotides used to purify (*i*) phage displaying the first three fingers of TFIIIA, (*ii*) phage displaying the three fingers of Zif268, and (*iii*) zinc finger phage from the phage display library. The Zif268 consensus operator sequence used in the x-ray crystal structure (13) is highlighted in *ii*, and in *iii*, where X denotes a base change from the ideal operator in oligonucleotides used to purify phage with new specificities. Biotinylation of one strand is shown by a circled B.

Despite this shortfall, it has been possible to isolate phage which bind with high affinity and specificity to given DNA sequences, demonstrating the remarkable versatility of the zinc finger motif.

Amino Acid–Nucleotide Base Contacts in Zinc Finger–DNA Complexes Deduced from Phage Display Selection. Of the 64 base triplets that could possibly form the binding site for variations of finger 2, we have so far used 32 in attempts to isolate zinc finger phage as described. Results from these selections are shown in Fig. 2. In general we have been unable to select zinc fingers which bind specifically to triplets without a 5' or 3' guanine, all of which return the same limited set of phage after three rounds of selection (see legend to Fig. 2). However, for each of the other triplets used to screen the library, a family of zinc finger phage is recovered. In these families, we find a sequence bias in the randomized α-helix, which we interpret as revealing the position and identity of amino acids used to contact the DNA. For instance, the middle fingers from the eight different clones selected with the triplet GAT (Fig. 2*d*) all have Asn at position +3 and Arg at position +6, just as does the first zinc finger of the *Drosophila* protein tramtrack, in which they are seen making contacts to the same triplet in the cocrystal with specific DNA (14). This indicates that the positional recurrence of a particular amino acid in functionally equivalent fingers is unlikely to be coincidental, but rather because it has a functional role. Thus, by using data collected from the phage display library (Fig. 2) it is possible to infer most of the specific amino acid–DNA interactions. Remarkably, most of the results can be rationalized in terms of contacts from the three primary α-helical positions (−1, +3, and +6) identified by x-ray crystallography (13) and database analysis (29).

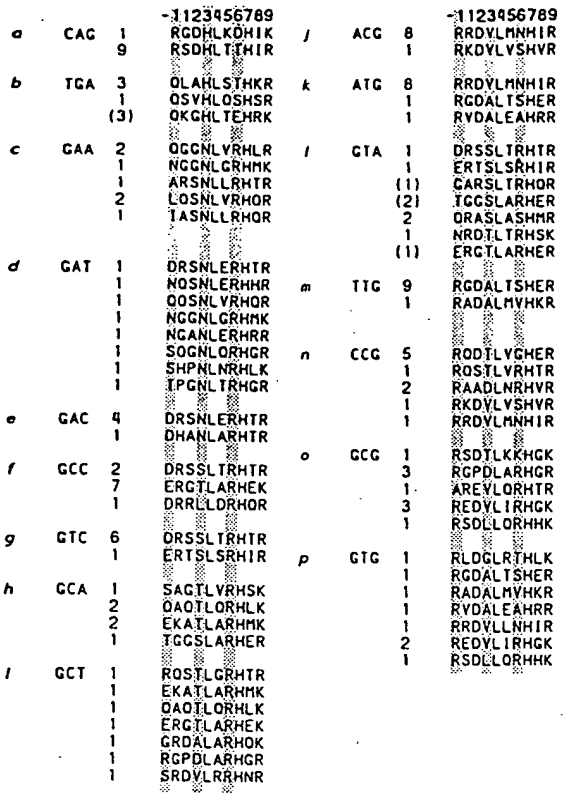

FIG. 2. Amino acid sequences of the variant α-helical regions from clones of library phage selected after three rounds using variants of the Zif268 operator. The amino acid sequences, aligned in the one-letter code, are listed alongside the oligodeoxynucleotides used in their purification (a–p). The latter are denoted by the sequence of the central DNA triplet in the "bound" strand of the variant Zif268 operator. The amino acid positions are numbered relative to the first helical residue (position +1), and the three primary recognition positions are highlighted. The accompanying numbers indicate the independent occurrences of that clone in the sequenced population (5–10 colonies); where numbers are in parentheses, the clone(s) was detected in the penultimate round of selection but not in the final round. In addition to the DNA triplets shown here, others were also used in attempts to select zinc finger phage from the library, but most selected two clones, one having the α-helical sequence KASNLVSHIR, and the other having LRHNLETHMR. Those triplets were ACT, AAA, TTT, CCT, CTT, TTC, AGT, CGA, CAT, AGA, AGC, and AAT.

As has been pointed out before (31), guanine has a particularly important role in zinc finger–DNA interactions. When present at the 5′ (Fig. 2 c–i) or 3′ (Fig. 2 m–o) end of a triplet, guanine selects fingers with Arg at position +6 or −1 of the α-helix, respectively. When present in the middle position of a triplet (Fig. 2b), guanine prefers His at position +3. Occasionally, guanine at the 5′ end of a triplet selects Ser or Thr at +6 (Fig. 2p). Since guanine can only be specified absolutely by Arg (32), this is the most common determinant at −1 and +6. We can expect this type of contact to be a bidentate hydrogen-bonding interaction as seen in the crystal structures of Zif268 (13) and tramtrack (14). In these structures, and in almost all of the selected fingers in which Arg recognizes G at the 3′ end, Asp occurs at position +2 to buttress the long Arg side chain (Fig. 2 o and p). When position −1 is not Arg, Asp rarely occurs at +2, suggesting that in this case any other contacts it might make with the second DNA strand do not contribute significantly to the stability of the protein–DNA complex.

Adenine is also an important determinant of sequence specificity, recognized almost exclusively by Asn or Gln which again are able to make bidentate contacts (32). When adenine is present at the 3′ end of a triplet, Gln is often selected at position −1 of the α-helix, accompanied by small aliphatic residues at +2 (Fig. 2b). Adenine in the middle of the triplet strongly selects Asn at +3 (Fig. 2 c–e), except in the triplet CAG (Fig. 2a), which selected only two types of finger, both with His at +3 (one being the wild-type Zif268, which contaminated the library during this experiment). The triplets ACG (Fig. 2j) and ATG (Fig. 2k), which have adenine at the 5′ end, also returned oligoclonal mixtures of phage, the majority of which were of one clone with Asn at +6.

In theory, cytosine and thymine cannot be reliably discriminated by a hydrogen-bonding amino acid side chain in the major groove (32). Nevertheless, cytosine in the 3′ position of a triplet shows a marked preference for Asp or Glu at position −1, together with Arg at +1 (Fig. 2 e–g). Asp is also sometimes selected at +3 and +6 when cytosine is in the middle (Fig. 2o) and 5′ (Fig. 2a) position, respectively. Although Asp can accept a hydrogen bond from the amino group of cytosine, we note that the positive molecular charge of cytosine in the major groove (33) will favor an interaction with Asp regardless of hydrogen-bonding contacts. However, cytosine in the middle position most frequently selects Thr (Fig. 2i) or Val or Leu (Fig. 2o) at +3. Similarly, thymine in the middle position most often selects Ser (Fig. 2l) or Ala or Val (Fig. 2p) at +3. The aliphatic amino acids are unable to make hydrogen bonds, but Ala probably has a hydrophobic interaction with the methyl group of thymine, whereas a longer side chain such as Leu can exclude thymine and pack against the ring of cytosine. When thymine is at the 5′ end of a triplet, Ser and Thr are selected at +6 (as is occasionally the case for guanine at the 5′ end). Thymine at the 3′ end of a triplet selects a variety of polar amino acids at −1 (Fig. 2d), and occasionally returns fingers with Ser at +2 (Fig. 2d) which could make a contact as seen in the tramtrack crystal structure (14).

Limitations of Phage Display. From Fig. 2 it can be seen that a consensus or bias usually occurs in two of the three primary positions (−1, +3, and +6) for any family of equivalent fingers, suggesting that in many cases phage selection is by virtue of only two base contacts per finger as is observed in the Zif268 crystal structure (13). Accordingly, identical finger sequences are often returned by DNA sequences differing by one base in the central triplet. One reason for this is that the phage display selection, being essentially purification by affinity, can yield zinc fingers which bind equally tightly to a number of DNA triplets and so are unable to discriminate. Second, since complex formation is governed by the law of mass action, affinity selection can favor those clones whose representation in the library is greatest even though their true affinity for DNA is less than that of other clones less abundant in the library. Phage display selection by affinity is therefore of limited value in distinguishing between permissive and specific interactions beyond those base contacts necessary to stabilize the complex. Thus, in the absence of competition from fingers which are able to bind specifically to a given DNA, the tightest nonspecific complexes will be selected from the phage library. Consequently, results obtained by phage display selection from a library must be confirmed by specificity assays, particularly when that library is of limited size.

Conclusion. The amino acid sequence biases observed within a family of functionally equivalent zinc fingers indicate that, of those α-helical positions randomized in this study, only three primary (−1, +3, and +6) positions and one auxiliary (+2) position are involved in the recognition of DNA. Moreover, a limited set of amino acids are to be found at those positions, and we presume that these make contacts

to bases. The indications therefore are that a code can be derived to describe zinc finger–DNA interactions. At this stage, however, although sequence homologies are strongly suggestive of amino acid preferences for particular base pairs, we cannot confidently deduce such rules until the specificity of individual fingers for DNA triplets is confirmed. We therefore defer making a summary table of these preferences until the following paper (34), in which we describe how randomized DNA binding sites can be used to this end.

While this work was in progress, a paper appeared by Rebar and Pabo (35) in which phage display was also used to select zinc fingers with new DNA-binding specificities. Those authors constructed a library in which the first finger of Zif268 was randomized, and screened with tetranucleotides to take into account end effects such as additional contacts from variants of this finger. Only four positions (−1, +2, +3, and +6) were randomized, chosen on the basis of the earlier x-ray crystal structures. The results of our work, in which more positions were randomized, to some extent justify Rebar and Pabo's use of the four random positions without apparent loss of effect. Moreover, randomizing only four positions decreases the theoretical library size so that full degeneracy can be achieved in practice. Nevertheless, we find that the results obtained by Rebar and Pabo by screening their complete library with two variant Zif268 operators are in agreement with our conclusions derived from an incomplete library. This again highlights the versatility of zinc fingers but, remarkably, both studies were unable to produce fingers which bind to the sequence CCT. It will be interesting to see whether sequence biases such as we have detected would be revealed if more selections were performed with Rebar and Pabo's library. In any case, it would be desirable to investigate the effects on selections of using different numbers of randomized positions in more complete libraries than we have used thus far.

Note Added in Proof. Since this paper was submitted, Jamieson *et al.* (36) have reported the use of random mutagenesis and phage display to alter the DNA-binding specificity of Zif268, in order to investigate DNA recognition properties.

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